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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07H 21/04, C07K 13/00, 17/02, C12N 1/21, 5/10, 15/70, G01N 33/543, 33/566, 33/577, 33/74

A1

(11) International Publication Number: WO 94/21665

(43) International Publication Date: 29 September 1994 (29.09.94)

(21) International Application Number: PCT/US94/03248

(22) International Filing Date: 24 March 1994 (24.03.94)

(30) Priority Data:
036,784 24 March 1993 (24.03.93) US

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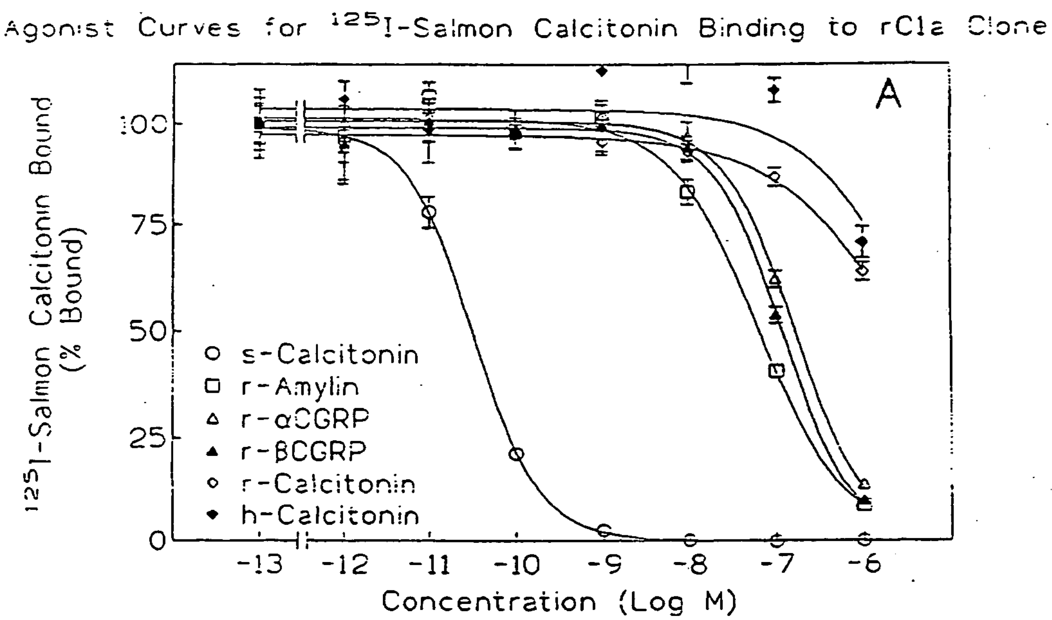
(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CLONED RECEPTORS AND METHODS FOR SCREENING



(57) Abstract

Cloned rat C1a and C1b receptors, nucleic acid encoding such receptors, and methods for identifying or screening or characterizing or assaying or isolating known or candidate calcitonin agonists or antagonists, including binding and selectivity assays utilizing preparations containing C1a or C1b receptors are described.

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CLONED RECEPTORS AND METHODS FOR SCREENING

Field of the Invention

The present invention relates to cloned receptors and to methods for screening for, identifying, isolating, characterizing, or quantitating physiologically active materials, such as chemical compounds, by assessing their ability to interact with receptor sites.

Description of Related Art

The following discussion is provided as background to the present invention.

Since its discovery over 30 years ago, the hypocalcaemic hormone calcitonin has been extensively investigated in both animals and man. Calcitonin is known to be a regulator of calcium homeostasis, acting principally on bone. It also has a direct action on the kidneys and on gastrointestinal secretory activity, as well as having both direct and indirect effects on the central nervous system. Azria, M, The Calcitonins: Physiology and Pharmacology (S. Karger AG, Basel 1989). Effects reported following intraventricular injection of salmon calcitonin include anorexia (Freed et al., Science 206:850-852, 1979), analgesia (Bragor et al., Life Sciences 22:971-978, 1978), decreased locomotor activity (Twery et al., Pharmocol. Biochem Behav. 18:857-862, 1983), and inhibition of gastric acid secretion (Morley et al., Science 214:671-673, 1981).

Although the actions of calcitonin on bone and its role in normal human bone physiology are still incompletely understood, current principal indications for the therapeutic use of calcitonin are for disorders

involving hypercalcemia, Paget's Disease (osteitis deformans), acute pancreatitis, and high-bone-turnover osteoporosis. It has also been used for the relief of bone pain associated with osteoporosis or bone metastases. Various calcitonins in use include natural porcine calcitonin, synthetic human calcitonin, synthetic salmon calcitonin, and a synthetic eel calcitonin analog.

Tissue-specific RNA processing of calcitonin gene transcripts leads to mRNAs encoding different peptide products, including precursors of calcitonin and the calcitonin gene-related peptides (α-CGRP and β-CGRP). In man, CGRP is distributed throughout the central nervous system. CGRP is also present in high concentrations in perivascular nerves throughout the body, including in the coronary and cerebral vessels. The most striking effect of CGRP is vasodilation, and in healthy volunteers infusion of CGRP causes hypotension and reflex tachycardia. CGRP is the principal circulating product of the human calcitonin gene, suggesting that it has an important physiological role in the control of blood flow and vascular tone. Like calcitonin, CGRP also inhibits bone resorption, gastric acid secretion and the perception of pain, albeit less potently. Currently, there are no established pharmaceutical uses for CGRP.

Amylin is a recently discovered peptide hormone that is co-secreted with insulin from the pancreas (see, e.g., Cooper et al., Diabetalogia 32:104, 1989; Cooper et al., Diabetes 88:493-496, 1989; Cooper et al., Biochim. Biophys. Acta 1014:247-258, 1989). Major metabolic effects of amylin reported in vivo include (1) a reduction in insulin-mediated glucose clearance (Molina et al. Diabetes

39:260-265, 1990, and Young et al., Am. J. Physiol. 259:457-461, 1990) and insulin-mediated suppression of hepatic glucose output (Molina et al. supra; Koopmans et al., Diabetes 39:101A, 1990), and, (2) an increase in plasma lactate and, subsequently, a sustained increase in plasma glucose. Similar actions of CGRP have subsequently been reported. It is also reported that amylin can exert certain other actions in vivo, including vasodilation (Brain et al., Am. J. Pathol. 136:487-490, 1990). Amylin, however, is 100- to 1000-fold less potent as a vasodilator than the related peptide CGRP. Amylin is also reported to lower plasma calcium in rabbits and rats (Datta et al., Biochem. Biophys. Res. Commun. 162:876-881, 1989), although human calcitonin is reported to be more effective than amylin in inducing hypocalcemia.

Principal therapeutic uses for amylin are those proposed for the treatment of diabetes. It has been determined that type-1 (insulin-dependent) diabetics, in addition to their life-threatening lack of insulin, have a marked amylin deficiency. The treatment of diabetes with amylin or agonists of amylin is described in Cooper, U.S. Patent No. 5,175,145, issued December 29, 1992, for "Treatment of Diabetes Mellitus With Amylin Agonists." It has also been proposed that excess amylin action contributes to the disordered metabolism in type 2 diabetes, glucose intolerance, insulin resistance and obesity, and the blockade of amylin with amylin antagonists has been identified as an appropriate therapeutic strategy for those conditions. Cooper et al., International Application No. PCT/US89/00049, "Treatment of Type 2 Diabetes Mellitus", published July 13, 1989; Cooper et al.,

International application No. 90307402.6, "Treatment of Obesity and Essential Hypertension and Related Disorders," published January 16, 1991.

Calcitonin and α-CGRP share common parentage in the calcitonin gene where alternative processing of the primary mRNA transcript leads to the generation of the two distinct peptides, which share only limited sequence homology (about 30% (Amara _et al._, _Science_, 229:1094-1097, 1985). The structure of amylin shows a 43% homology to α-CGRP, a 46% homology to β-CGRP, and some similarity to insulin. Amylin may be one member of a family of related peptides which include CGRP, insulin, insulin-like growth factors, and the relaxins and which share common genetic heritage (Cooper _et al._, _Prog. Growth Factor Research_ 1:99-105, 1989).

It is believed that calcitonin and the related peptides CGRP and amylin act via membrane receptors at least some of which serve to activate adenylate cyclase and generate cyclic AMP as an intracellular second messenger. Young et al. have shown that amylin works in skeletal muscle via a receptor-mediated mechanism that promotes glycogenolysis, by activating the rate-limiting enzyme for glycogen breakdown, phosphorylase a (Young _et al._ 281 _FEBS Lett._ 149, 1991). Amylin receptors and receptor preparations are described in Beaumont et al., International Application No. PCT/US92/02125, "Receptor-Based Screening Methods for Amylin Agonists and Antagonists," published October 1, 1992. Binding studies included therein using rat brain amylin receptor preparations showed that rat amylin was the most potent binding compound tested. Eel and salmon calcitonin exhibited slightly less potent binding to the amylin

receptor. Rat and human β-CGRP were 3-fold and 5-fold less potent than amylin, respectively, while rat and human α-CGRPs were some less potent than the β-CGRPs. Rat calcitonin was a very weak inhibitor, indicating that the amylin receptor does not respond to the calcitonin circulating in the rat. The higher affinity of amylin than CGRP for this receptor correlates with the relative potencies of these peptides at inhibiting glycogenolysis in soleus muscle.

Binding sites for calcitonin and CGRP are widely distributed in the central nervous system. However, the two peptides act at their own distinct high affinity receptors with distinct biochemical specificities and little interaction at the alternate receptor site. For example, it has been reported that in both human (Tschopp et al., Proc. Nat. Acad. Sci. USA 82:248-252, 1985) and rat (Sexton et al., Neuroscience 19:1235-1245, 1986) cerebral cortex, calcitonin was 500- to 1000-fold less potent than either rat or human CGRP in competition for CGRP binding sites. Similarly, CGRP was 500- to 1000-fold less potent in competing for calcitonin sites in both brain and renal membranes (Goltzman and Mitchell, Science 227:1343-1345, 1985), as well as in whole kidney sections (Sexton et al., Kidney Int. 32:862-868, 1987). Some data regarding atypical CGRP binding sites in regions of the rat brain have also been reported (Dennis et al., Soc. Neurosci. Abs. 16:514, Abstract 220.7, 1990; Sexton et al., Neurochem. Int. 12:323-335, 1988). The presence and distribution of calcitonin receptors in the central nervous system have been described previously (Henke, Tobler, Fischer, Brain Res. 272:373, 1983). Structural requirements for binding

to central calcitonin receptors differ from those required to produce hypocalcemia, suggesting separate central and peripheral calcitonin receptor subtypes.

SUMMARY OF THE INVENTION

Applicant has discovered, isolated and cloned two receptors that bind calcitonin and related peptides. These receptors are herein termed the C1a receptor and the C1b receptor. By C1a receptor is meant a receptor of the amino acid sequence shown as C1a in Fig. 1, i.e., the deduced 479 amino acid sequence of cDNA clone L2175-D20, and any functional homolog thereof. By C1b receptor is meant a receptor of the amino acid sequence shown as C1b in Fig. 1, i.e., the deduced 516 amino acid sequence of the cDNA clone U3237-A2, and any functional homolog thereof. By "functional homolog" is meant a receptor having variation(s) in amino acid sequence while retaining C1a or C1b binding activity, respectively.

The deduced amino acid sequence of the C1a receptor is 78% and 66% homologous with the human and pig calcitonin receptors, respectively. The amino acid sequence of the C1b receptor is identical to the C1a receptor except for a 37 amino acid insert in the second extracellular domain between transmembrane domains 2 and 3.

Northern analysis indicates that C1a and C1b receptor transcripts are present in brain and kidney. When assessed by more sensitive PCR methods, C1a and C1b sequences were also amplified from skeletal muscle and lung mRNA. Neither was detected in ovary, pancreas or liver.

Receptor binding studies indicate that both C1a and C1b receptors have high affinity (5-50 pM) for salmon

calcitonin, low affinity for rat amylin and the rat calcitonin gene related peptides, and lower affinity for rat and human calcitonin.

The Cla and Clb receptors are useful in methods for identifying calcitonin agonist or antagonist compounds useful in the treatment of various disease states or conditions, such as obesity, anorexia or pain. These receptors are also useful in methods for identifying receptor selectivity characteristics of amylin agonist or antagonist compounds useful in the treatment of various disease states or conditions, such as diabetes mellitus, impaired glucose tolerance, and insulin resistance.

The Cla and Clb receptors are useful in the screening procedures detailed below, as well as those described (with respect to amylin receptors) in Beaumont, U.S. Serial No. 07/670,231, and those described (with respect to myotonin receptors) in Beaumont, U.S. Serial No. 07/821,731 the disclosures of which are hereby incorporated by reference. For example, the invention features rapid, inexpensive and physiological methods for identifying, screening and characterizing potential compounds useful for treatment of diseases or conditions characterized by an elevated or undesired level of amylin activity (in the case of antagonists) and conditions which are benefitted by amylin (in the case of agonists). The methods include assessing the ability of candidate molecules to compete against tracer concentrations of certain labeled peptides, including certain labeled peptide hormones and fragments and analogs thereof, for binding to Cla or Clb receptor binding sites. By way of example, the Cla and Clb binding

sites may be present in transfected cells, or in membranes prepared or isolated from said cells.

Thus, in one aspect, the invention provides an assay method for identifying or screening for calcitonin agonist or antagonist compounds. The method includes bringing together a test sample and a Cla or Clb receptor preparation. The test sample contains one or more test compounds, and the Cla or Clb receptor preparation contains a Cla or Clb receptor protein capable of binding to a Cla or Clb receptor-binding compound. The test sample is incubated with the receptor preparation under conditions that allow binding to the Cla or Clb receptor protein. Those test samples containing one or more test compounds which detectably bind to the Cla or Clb receptor protein are then identified.

In preferred embodiments, this method further includes the steps of screening test samples which detectably bind to a Cla or Clb receptor for in vitro or in vivo stimulation or inhibition of calcitonin receptor-mediated activity, and identifying those test samples which act as receptor agonists or antagonists at either receptor.

In other preferred embodiments, test samples which detectably bind to a Cla or Clb receptor protein are identified by measuring the displacement of a labeled first ligand from the receptor preparation by the test sample, and comparing the measured displacement of the first labeled ligand from the receptor preparation by the test sample with the measured displacement of the labeled first ligand from the receptor preparation by one or more known second ligands. Labeled first ligands and second ligands include salmon calcitonin, rat amylin, and rat CGRP.

Useful receptor preparations include transfected cells bearing a C1a or C1b receptor, membrane preparations bearing a C1a or C1b receptor, and isolated C1a or C1b receptor protein. Test samples used in any of the above methods that contain more than one test compound and which yield positive results can then be divided and retested as many times as necessary, and as appropriate, to identify the compound or compounds in the test sample which are responsible for yielding the positive result.

In particularly preferred embodiments, the first ligand is labelled with a member selected from the group consisting of radioactive isotopes, nonradioactive isotopes, fluorescent molecules, chemiluminescent molecules, and biotinylated molecules; the known second ligand or ligands are selected from the group consisting of an amylin, a calcitonin, an α-CGRP, and a β-CGRP (e.g., human amylin, dog amylin, rat amylin, eel calcitonin, salmon calcitonin, human α-CGRP, human β-CGRP, rat α-CGRP, and rat β-CGRP) and the test sample comprises one or more known or unknown test compounds. One or more non-specific C1a or C1b binding sites which may be present on cells that do not comprise a desired or target receptor may optionally be blocked.

In another aspect, the invention provides an assay method for evaluating one or more receptor binding characteristics sought to be determined for a known or a candidate C1a or C1b agonist or antagonist compound. The method includes the steps of assessing or measuring the ability of the compound to compete with a labeled ligand for binding to a C1a or C1b receptor preparation, as described above; assessing or measuring the ability of the

compound to compete against the labeled ligand for binding to a CGRP or amylin receptor preparation, or assessing or measuring the ability of the compound to compete against the labeled ligand for binding to more than one or all of said receptor preparations; and, determining the receptor binding characteristic sought to be determined for the compound. Receptor binding characteristics which may be determined include binding affinity and binding specificity. CGRP receptor preparations include primary cell cultures or established cell lines e.g., the SK-N-MC cell line and the L6 cell line. Amylin receptor preparations include cell or membrane preparations, e.g., such as those described in Beaumont et al., supra.

Similarly, C1a and C1b receptor preparations may be used in selectivity assays or screens in which, for example, an amylin agonist or antagonist compound is tested for C1a or C1b receptor binding. By using such assays, amylin agonists or antagonists having a preferred receptor binding profile may be identified and selected. In other words, molecules that bind to the C1a and C1b receptor, or which have certain receptor binding potency, can be selected or rejected as candidate therapeutics based on such assay results.

In still another aspect, the invention provides an assay method for determining the presence or amount of a C1a or C1b receptor binding compound in a test sample to be assayed. The method includes the steps of bringing together the test sample and a C1a or C1b receptor preparation, as described above; measuring the ability of the test sample to compete against a labeled ligand for binding to the C1a or C1b receptor preparation; and,

optionally, relating the amount of C1a or C1b receptor binding compound in the test sample with the amount of C1a or C1b receptor binding compound measured for a negative control sample, the negative control sample being known to be free of any C1a or C1b receptor binding compound, and/or relating the amount of C1a or C1b receptor binding compound in the test sample with the amounts of C1a or C1b receptor binding compound measured for positive control samples which contain known amounts of C1a or C1b receptor binding compound, in order to determine the presence or amount of C1a or C1b receptor binding compound present in the test sample.

This assay method, in still further embodiments, can be utilized to evaluate the stability, potency or solubility characteristics of C1a or C1b binding ligand preparation, such as a calcitonin preparation. In preferred embodiments, a C1a or C1b receptor binding compound and/or labelled ligand is an calcitonin agonist or a calcitonin antagonist; and the test sample is a biological fluid, selected from the group consisting of blood, plasma, urine, cerebrospinal fluid, lymph fluid, or a calcitonin preparation; and the assay method includes evaluation of the stability, potency or the solubility of a calcitonin preparation.

In another aspect, the receptor preparations of the invention can be utilized to prepare anti-C1a or anti-C1b receptor antibodies, including polyclonal antisera and monoclonal antibodies, utilizing art-known methods.

In another aspect, the invention is used to screen cell lines, cells from tissue, and cells from human or

animal blood in order to identify those which carry Cla or Clb receptors.

The Cla or Clb receptor preparations of the invention may also be bound to a solid phase and used in various affinity chromatography methods and used, for example, for the purification of peptides such as a calcitonin, or the evaluation of samples known or suspected to contain calcitonin or calcitonin agonists or antagonists.

In other aspects, the invention features purified Cla or Clb receptor; and purified nucleic acids encoding such calcitonin receptors, e.g., by standard techniques. By "purified" is meant that the Cla or Clb receptor or nucleic acid encoding it is separated from its natural environment, preferably as a homogeneous preparation having at least 60-90% by weight of the desired product.

It is thus an object of this invention to identify receptor preparations suitable for the various screening methods of this invention.

It is another object of this invention to provide details of the screening methods of the invention as applied to potential Cla or Clb agonists and antagonists.

It is still another object of this invention to teach the method for assessing the relative potencies and specificities of the candidate Cla and Clb agonists and antagonists.

It is still another object of the invention to provide a method, using Cla or Clb receptor preparations, for determining the binding and/or binding potency of other receptor binding compounds, such as amylin agonists and antagonists, in a receptor selectivity screening assay.

These and other objects will become readily apparent by reference to the specification and the appended claims.

LEGEND TO FIGURES

The drawing will first briefly be described.

Drawing

Figure 1 is a depiction of the complete amino acid sequences of a C1a receptor and a C1b calcitonin receptor, and putative receptor domains have been assigned. TM refers to the transmembrane portions of the receptors, I to the intracellular portions, and E to the extracellular portions. The receptors appear to have 7 transmembrane regions, 4 intracellular domains, and 4 extracellular domains. The amino-terminus domain is extracellular (E1) and the carboxy-terminus domain is intracellular (I4). Dashes indicate gaps in the sequences introduced to optimize homology. The standard one-letter abbreviations for amino acids have been used: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Figure 2 is a graphical representation of the saturation isotherm of ^{125}I-salmon calcitonin binding to C1a receptor membranes. Amount of bound ^{125}I-salmon calcitonin (in pmol/mg) is plotted against ^{125}I-salmon calcitonin receptor concentration (pM).

Figure 3 depicts a Scatchard plot of C1a receptor binding.

Figure 4 is a graphical representation of the saturation isotherm of ^{125}I-salmon calcitonin binding to Clb receptor membranes. Amount of bound ^{125}I-salmon calcitonin (in pmol/mg) is plotted against ^{125}I-salmon calcitonin receptor concentration (pM).

Figure 5 depicts a Scatchard plot of Clb receptor binding.

Figure 6 is a graphical representation of competition studies for the Cla receptor. Data were fit to a 4-parameter logistic equation, using the Cheng-Prusoff relationship to derive apparent inhibition constants (Ki) from IC_{50} values. Values shown represent means $\pm$ standard deviations from duplicate experiments. Percent bound ^{125}I-salmon calcitonin is plotted against concentrations of the unlabeled ligands salmon calcitonin (open circle), rat amylin (open square), rat and CGRP (open triangle), rat β-CGRP (filled triangle), rat calcitonin (open diamond) and human calcitonin (filled diamond).

Figure 7 is a graphical representation of competition studies for the Clb receptor. Data were fit to a 4-parameter logistic equation, using the Cheng-Prusoff relationship to derive apparent inhibition constants (Ki) from IC_{50} values. Values shown represent means $\pm$ standard deviations from duplicate experiments. Percent bound ^{125}I-salmon calcitonin is plotted against concentrations of the unlabeled ligands salmon calcitonin (open circle), rat amylin (open square), rat and CGRP (open triangle), rat β-CGRP (filled triangle), rat calcitonin (open diamond) and human calcitonin (filled diamond).

Figure 8 shows cyclic AMP production in COS cells transiently transfected with the Cla or Clb receptor.

Cells were incubated with buffer alone (filled bars), 1 μM salmon calcitonin (shaded bars), or 10 μM isoproterenol (cross-hatched bars). Values are means $\pm$ standard deviation (n=8).

DETAILED DESCRIPTION OF THE INVENTION

The C1a and C1b receptors of the present invention were cloned using the technique of mixed oligonucleotide-primed amplification of cDNA (MOPAC). In this technique, degenerate oligonucleotide primers corresponding to conserved regions of potentially related sequences are used to amplify homologous sequences by the polymerase chain reaction (PCR) from cDNA prepared from RNA of tissues or cell lines. Specifically, mixtures of oligonucleotides (A312 and A313) corresponding to conserved regions in TM3 and TM7 of the pig calcitonin, opossum PTH/PTHrP, and rat secretin G-protein coupled receptors were used as primers in an attempt to amplify amylin receptor sequences from rat nucleus accumbens poly(A)$^+$ RNA. PCR amplified products were analyzed by Southern blotting with an oligonucleotide probe (A315) corresponding to a third conserved region of the calcitonin and PTH/PTHrP receptors. The PCR product was gel purified and subjected to a second round of amplification. The secondary PCR product was subcloned into pCR1000 and sequenced.

An oligo(dT)-primed rat nucleus accumbens cDNA library was subsequently screened for a full-length cDNA sequence corresponding to the PCR product. The cDNA library was non-directionally cloned in eukaryotic expression vector pcDNAI and subsequently transformed into *E. coli* MC1061/P3. Plasmid DNA was prepared from 72 pools each representing

~5000 individual library transformants. These plasmid DNA pools were used as template in a PCR amplification with the A312 and A313 degenerate primer mixtures and products were analyzed by Southern blotting and probing with the A315 oligonucleotide. Ultimately, a PCR product ~470 bp that hybridized with A315 was amplified in 4 of the 72 pools examined. The bacteria corresponding to these pools were screened by colony hybridization using A315 as a probe and an isolated bacterial transformant was isolated from 2 of the 4 pools. Restriction endonuclease analysis indicated that both rat nucleus accumbens cDNA library clones (pcDNAI-175 and pcDNAI-237) were about 2.5 kb in length.

The 5' RACE (rapid amplification of cDNA ends) technique (Frohman et al., Natl. Acad. Sci. USA 85:8998-9002, 1988; Frohman, M.A. PCR Protocols: A Guide to Methods and Applications (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., eds.) pgs. 28-38, Academic Press, San Diego (1990); Frohman, M.A. *Amplifications* 5:11-15, 1990; Schuster et al., Focus 14:46-52, 1992) was employed to amplify the unknown sequence at the 5' end of the receptor transcripts in order to construct full-length cDNAs. Oligonucleotide A333 was used to prime 1st strand cDNA synthesis from rat nucleus accumbens poly(A)$^{+}$ RNA. Oligonucleotide A361, from the 3rd extracellular loop was used as the second gene-specific primer for PCR amplification. Product bands were isolated, subcloned and partially sequenced. Full-length cDNA for the C1a receptor was constructed by ligating corresponding 5' RACE (L2) and cDNA library (175) sequences into eukaryotic expression vector pcDNAI. Full-length cDNA construct pcDNAI-L2175-D20 contains a ~3.4 kb insert in the correct orientation for

expression. Full-length cDNA for the Clb receptor was similarly constructed by ligating corresponding 5'RACE (U3) and cDNA library (237) sequences into eukaryotic expression vector pcDNAI. Full-length cDNA construct preDNAI-U3237-A2 contains a ~3.5 kb insert in the correct orientation for expression. The unique StuI site in the 3rd intracellular domain was restored in both constructs indicating regeneration of the correct reading frame at the only site within the protein coding region used for construction of the full-length molecules from the 5' RACE and cDNA products.

The present invention provides novel inexpensive, rapid and physiological methods for screening, identifying, and characterizing potential agonists and antagonists at two novel receptors, the Cla receptor and the Clb receptor, as well as the use of these receptors to identify calcitonin agonists and antagonists, and in agonist and antagonist receptor selectivity screening assays. This includes assessing the relative abilities of candidate agonists and antagonists to compete against relevant peptides for binding to specific Cla or Clb receptor sites. The receptor sites used for these and other purposes may be present as isolated receptor-bearing tissues, cells prepared from said receptor bearing tissues, transfected cells expressing said receptor sites, membrane preparations derived from said prepared or transfected cells, or isolated receptor protein preparations, including cloned receptor preparations using recombinant DNA techniques.

A Cla or Clb receptor assay can be used to determine the concentration of Cla receptor- or Clb receptor-active compounds in unknown solutions or mixtures. Cla and Clb

receptors are assayed as described below. For example, a membrane or cell preparation containing a high density of C1a or C1b receptors is incubated with, for example, radiolabeled calcitonin and unlabelled calcitonin. In this manner, a competition curve is generated relating the amount of calcitonin in the assay tube to the inhibition of radiolabelled calcitonin binding produced. In additional tubes, unlabelled peptide is replaced by a solution containing an unknown amount of calcitonin to be quantified. This solution may be plasma, serum or other fluid, or solid mixture dissolved in assay buffers. The unknown solution is preferably added in a volume of less than or equal to about 10% of the final assay volume, so as not to significantly alter the ionic content of the solution. If larger volumes of unknown are used, a solution containing an equivalent salt content is included as a control for effects of altered ionic content on binding. Nonspecific binding, i.e., binding of radiolabelled calcitonin in the presence of a high concentration (10^{-6}M) of unlabelled calcitonin, is subtracted from total binding for each sample to yield specific binding. The amount of inhibition of specific binding of radiolabelled calcitonin produced by the unknown is compared to the inhibition curve produced by unlabelled calcitonin in order to determine the content of calcitonin or calcitonin receptor-active substances in the unknown sample. Methods for performing these calculations are described in several sources, such as in Neurotransmitter Receptor Binding, eds H. Yamamura, S.J. Enna, and M.J. Kuhar (Raven Press, New York, 1991).

This method is used to quantitate the amount of C1a or C1b receptor active compounds in a known or an unknown sample, and may be used to quantitate C1a or C1b receptor active compounds in plasma or other body fluids and tissues, for use in identifying active metabolites, pharmacokinetics, stability, solubility, or distribution of C1a or C1b receptor agonists and antagonists, calcitonin agonists and antagonists, and amylin agonists and amylin antagonists. It may also be used to identify, isolate and purify peptides having a high affinity for the C1a or C1b receptor.

A C1a or C1b receptor can also be used in a high throughput screen, optionally utilizing robotic systems such as those known in the art, for identifying compounds which displace, for example, calcitonin from either receptor and, thus, for example, for identifying candidate C1a, C1b, and calcitonin agonists or antagonists. The assay can be used to screen, for example, libraries of synthetic compounds, extracts of plants, extracts of animal tissue, extracts of marine organisms, or bacterial or fungal fermentation broths.

In one embodiment, an initial step brings together a C1a or C1b receptor preparation, pre-incubated with radiolabelled calcitonin and a solution of test compound. For organic extracts, the final concentration of solvent should generally not exceed that which displaces the standard displacement curve of labelled calcitonin by cold calcitonin by 25%, i.e., shifts the measured IC_{50} by less than 25%. This can be evaluated for each selected solvent. For identified compounds from synthetic libraries, the test concentration will be about 100nM, 1μM, or 10μM depending

on the frequency with which positive tests occur. A positive will typically be represented by at least about a 20% reduction of specific binding of labelled calcitonin. With broths and extracts, a positive test will be denoted by at least about 20%, 50% or 80% reduction in specific calcitonin binding, according to the frequency of positive tests.

It is useful in high throughput screening to check compounds or mixtures giving a positive test in an initial screen for non-specific interference with ligand binding. In a preferred embodiment, all positive testing compounds or extracts are exposed to a binding assay for another ligand in the same membrane preparation. A suitable assay for evaluating non-specific effects will be a radiolabelled standard reagent for determination of binding to a standard receptor in the vas deferens or tissue being used. Those receptors which are relatively abundant in the tissue and readily assayed should be chosen. Any compound, broth, or extract that tests positive in a Cla or Clb receptor screen, and which also tests positive by the same quantitative criteria in the standard receptor screen is rejected as non-selectively interfering with ligand binding to membrane receptors.

For compounds meeting the described criteria, the potency of interaction with a Cla or Clb receptor and, if relevant, the amylin, CGRP and/or myotonin receptors, are determined by measuring the displacement of ligand from the membrane preparations by a range of concentrations of the test compound. With mixtures of unknown compounds, as in broths and extracts, the desired activity is isolated and purified by art-known methods including HPLC, followed by

testing the separated materials to determine which retain the desired activity. When pure or relatively pure active material is obtained, its potency at a Cla or Clb, an amylin, a CGRP, myotonin or other receptor can also be determined. Art-known methods including NMR, mass spectroscopy, and elemental analysis may be used to make a chemical identification of any isolated material having the desired receptor binding activities.

At any desired stage following identification of selective displacement from Cla or Clb receptors, a positive testing material can be assessed in a functional assay to assess calcitonin agonist or antagonist activity. For example, calcitonin agonist may be determined through inhibition of insulin-stimulated incorporation of labelled glucose into glycogen in rat soleus muscle. The material can also be tested for calcitonin antagonist activity in this assay by assessing its ability to restore insulin-stimulated incorporation of labelled glucose into glycogen in rat soleus muscle incubated with 10, 20, 50 or 100 nM rat amylin. Calcitonin agonist activity can also be assessed by measuring hypocalcemia, analgesia, or anorexia following <u>in vivo</u> administration (<u>e.g.</u>, Zaidi, M., <u>et al</u>., <u>Exper</u>. <u>Phys</u>. 75:529-536, 1990). Antagonist activity is measured by assessing the ability of the test compound to block these actions of calcitonin. Also, by applying different concentrations of the test material in these assays, the potency of Cla, Clb, or calcitonin agonist or antagonist action can be determined.

In other embodiments, for assessment of whether materials testing positive in a Cla or Clb receptor binding assay are agonist or antagonists, the test materials are

brought together with Cla or Clb responsive membrane or cell systems in which calcitonin changes rates of synthesis of cyclic AMP (cAMP). Such preparations include membranes prepared from cultured or transfected cell lines with abundant Cla or Clb or amylin receptors, or the cells themselves. Changes in cAMP levels are measured by radioimmunoassay following exposure of the membrane or cell preparations, incubated according to art-known methods. Materials testing positive in displacing calcitonin from Cla or Clb receptors and having no effect on cAMP production can be expected to be antagonists. Antagonist action can be further evaluated by incubating various concentrations of the material with calcitonin or a calcitonin agonist and measuring the degree of inhibition of the changes in cAMP evoked by the calcitonin or calcitonin agonist.

The invention can also be used to screen cell lines, cells disaggregated from tissue, and cells from human or animal blood for Cla, Clb, or calcitonin receptors. These cells will be used as a readily available source for additional Cla or Clb receptor preparations for development of agonists and antagonists of calcitonin. Membranes from cells are obtained by homogenization of cells with an instrument such as Polytron (Brinkman Instruments) followed by centrifugation. Membranes so obtained are combined with, e.g., ^{125}I-salmon calcitonin, in a buffer system such as that described in the examples below, and are incubated and collected as described in those examples. Specific binding of ^{125}I-salmon calcitonin to the cell membrane is identified by measuring the decrease in binding obtained in the presence of, for example, 10^{-7} M salmon calcitonin.

Cells in which there is a significant difference between total binding (triplicate tubes) and nonspecific binding (triplicate tubes) at the $P<0.05$ level will be used for further study of C1a or C1b receptor function.

Subcellular membrane fractions obtained by differential or density gradient centrifugation are assayed for specific binding of radiolabelled calcitonin in order to identify the membrane fraction containing the highest density of C1a or C1b receptors per milligram protein (as assayed by Bradford or Lowry protein assays). The membrane fraction with highest receptor density is preferably used for further purification.

This membrane fraction is collected and treated in a buffered solution with several membrane solubilizing agents, including triton, digitonin, octyl glucoside, deoxycholate, and cholate, at concentrations of from 0.001% to 1% detergent at reduced temperature (4°C) for about 1 hour. Protease inhibitors (including phenylmethylsulfonyl fluoride, EDTA, aprotinin) are included in the buffer system to prevent receptor degradation during or after solubilization. After treatment of membranes with detergents, unsolubilized membranes are sedimented by centrifugation at high speed (100,000 x g for 1 hour) and resulting supernatants containing solubilized receptors are assayed for binding of radiolabelled calcitonin as described above. Solubilized receptors can be collected by filtration on polyethyleneimine-coated filters (Bruns et al. Anal. Biochem. 132:74-81, 1983). Alternatively, solubilized receptors are collected by methods such as precipitation with polyethyleneglycol, gel filtration, or equilibrium dialysis. Binding characteristics (such as

affinity for amylin, CGRP and calcitonins) of solubilized receptors are assessed and should match the characteristics of membrane-localized receptors.

After determining conditions suitable for solubilizing C1a or C1b receptors and for assaying solubilized receptors, these solubilized receptors are purified away from other solubilized membrane proteins by chromatographic procedures, such as affinity chromatography on supports to which calcitonin has been coupled, ion exchange chromatography, lectin agarose chromatography, gel filtration, and hydrophobic interaction chromatography. Chromatography column eluates are tested for specific C1a or C1b receptor binding to protein content, in order to identify peaks containing receptors and the extent of purification. Before inclusion in the final purification protocol, each chromatographic step is tested to determine the extent to which it contributes to receptor purification, as measured by an increase in specific radiolabelled calcitonin or amylin binding per milligram protein. Desired chromatography steps are combined sequentially, using large quantities of starting material, in order to obtain partially or completely purified receptors, as desired.

Receptors, for example, those which have been partially or completely purified by this method may be used to generate C1a or C1b receptor-specific antibodies for use in diagnosis (disease states with altered receptor density, distribution, or antigenicity) and for use in screening, for example, tissues or recombinant libraries for C1a or C1b receptor expression. The C1a or C1b receptor sequences

can also be used to probe for other Cla or Clb receptor-encoding gene sequences by art-known methods.

Specific embodiments of the receptors and the receptor binding assay and screening methods of this invention are exemplified in the following Examples. These Examples are not to be interpreted as limiting the scope of the invention in any way, the scope being disclosed in the entire specification and claims.

EXAMPLE 1

PREPARATION OF OLIGONUCLEOTIDES

A pair of degenerate oligonucleotides corresponding to conserved regions of the calcitonin, PTH/PTHrP, and secretin receptors were synthesized for use in MOPAC. A312 is a mixture of 16 18-mers corresponding to the transmembrane (TM) region 3 sense strand: 5'-GA(A/G)GG(G/C)(G/C)TCTA(C/T)CTTCAC-3'. A313 is a mixture of 64 17-mers corresponding to the TM7 antisense strand: 5'-(T/C)(C/G)(A/G)TTG(C/A)(A/G)GAA(G/A)CAGTA-3'. A third degenerate oligonucleotide, designated A315, corresponding to a different TM7 region than above conserved in the calcitonin and PTH/PTHrP receptors was used as a hybridization probe: 5'-GCAACGAA(G/A)AATCCCTGGAA-3'.

From partial DNA sequence information, two oligonucleotides specific to the novel receptors described herein were also synthesized: A333 (TM7 antisense strand; positions 1372-1392 of clone L2175-D20) 5'-CCCTGGAAATGAATCAGAGAG-3'; and, A361 (4th extracellular domain antisense strand; positions 1342-1363) 5'-$(CAU)_4$ATAATCATAGATCTTCCCAAGC-3'.

Oligonucleotides were synthesized on an Applied Biosystem (Foster City, CA) model 381A DNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry. Following synthesis they were cleaved from the support, deprotected and eluted. After evaporation to dryness they were dissolved in water.

EXAMPLE 2

PREPARATION OF RNA

Fresh nucleus accumbens, cerebellum, liver, soleus muscle, and gastrocnemius muscle tissue samples were obtained from rats and immediately frozen in liquid N_2 and then stored at -80°C. Frozen tissue was pulverized to a fine powder using a porcelain mortar and pestle immersed in a bath of liquid N_2. Poly(A)$^+$ RNA was isolated from powdered tissue samples by a guanidinium isothiocyanate procedure and oligo (dT) cellulose affinity chromatography (Fast Track; Invitrogen, San Diego, CA) according to the manufacturer's instructions.

Poly(A)$^+$ RNA from rat ovary, pancreas, skeletal muscle, smooth muscle, and kidney was obtained from Clonetech, Palo Alto, CA.

EXAMPLE 3

AMPLIFICATION OF RNA

Reverse transcription of poly(A)$^+$ RNA to cDNA and subsequent complification by the PCR (RT-PCR) was accomplished using reagents from the GeneAmp RNA PCR kit (Perkin Elmer Cetus; Norwalk, CT). 50 ng of rat nucleus accumbens poly(A)$^+$ RNA was reverse transcribed in a final 20 μl volume containing 10 mM Tris-HCl, pH 8.3/50 mM KCl/5

mM $MgCl_2$/ 1 mM each dNTP/2.5 μM random hexamer primers/1 unit μl^{-1} RNase inhibitor/2.5 units μl^{-1} Moloney murine leukemia virus reverse transcriptase for 10 min at 22°C followed by 45 min at 42°C. The reaction was terminated by heating for 5 min at 99°C followed by chilling to 4°C. The above 20 μl reaction was adjusted to a final 100 μl volume containing 10 mM Tris-HCl, pH 8.3/50 mM KCl/2 mM $MgCl_2$/2 μM each upstream (A312) and downstream (A313) degenerate oligonucleotide primers/0.2 mM each dNTP (contributed from the reverse transcription reaction) containing 2.5 units AmpliTaq DNA Polymerase (Perkin Elmer Cetus; Norwalk, CT). 50 PCR cycles involving denaturation for 1 min at 95°C, annealing for 1 min at 40°C, and primer extension for 1 min at 72°C were run in a Perkin Elmer Cetus DNA thermal cycler and followed by a final extension at 72°C for 10 min.

EXAMPLE 4

SOUTHERN BLOTTING

RT-PCR products were separated on 1% agarose/3% NuSieve gels and visualized by ethidium bromide staining. Gels were denatured for 30 min in 0.4 N NaOH/0.6 M NaCl with gentle agitation and then neutralized in 1.5 M NaCl/0.5 M Tris-HCl, pH 7.5 for 30 min with gentle agitation. Capillary transfer to GeneScreen Plus nylon support (Dupont; Boston, MA) with 10X SSC (1X SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) wicking buffer proceeded overnight. The blot was then immersed in 0.4 N NaOH for 1 min to ensure complete denaturation of the transferred DNA, briefly neutralized in 0.2 M Tris-HCl, pH 7.5/2X SSC, and air dried.

Blots were prehybridized in 6X SSPE (1X SSPE = 0.18 M NaCl/10 mM $NaPO_4$, pH 7.7/1 mM EDTA)/1% SDS/5X Denhardt's (1X Denhardt's = 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin) at 42°C. Blots were subsequently hybridized with ^{32}P-labeled A315 oligonucleotide in 6X SSPE/1% SDS/2X Denhardt's at 42°C overnight. Blots were washed in 1X SSPE/1% SDS at 37°C and exposed to Kodak XAR film with an intensifying screen at -80°C.

EXAMPLE 5

ISOLATION OF DNA FRAGMENTS FROM PCR REACTIONS OR GELS

PCR products were isolated from 3% NuSieve agarose gels by melting excised gel bands at 65°C and digesting the agarose with β-agarase I (New England BioLabs; Beverly, MA) according to the manufacturers instructions. After ethanol precipitation the products were dissolved in dH_2O. Alternatively, DNA fragments were isolated from low melting point agarose gel slices or directly from PCR reactions using a DNA purification resin (Magic PCR preps DNA purification system; Promega, Madison, WI) according to the manufacturer's instructions. 1-10 ng of gel isolated PCR products were re-amplified under the conditions described in Example 3 above for 40 cycles.

EXAMPLE 6

SUBCLONING DNA FRAGMENTS

PCR amplified DNA fragments were ligated to plasmid vector pCR1000 (TA Cloning System; Invitrogen, San Diego, CA) and used to transform *E. coli* INVαF' according to the manufacturer's protocol. Transformants harboring inserts

were identified by colony hybridization screening (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) using oligonucleotide A315 as a ^{32}P-labeled probe. The prehybridization and hybridization conditions were the same as described in Example 4 above for the Southern blot analysis.

EXAMPLE 7

cDNA LIBRARY SCREENING

An oligo(dT)-primed and size-selected (>800 bp) Wistar rat nucleus accumbens cDNA library was bidirectionally cloned in eukaryotic expression vector pcDNAI (Invitrogen; San Diego, CA) and transformed into *E. coli* MC1061/P3. Plasmid DNA was prepared from 72 sublibrary pools of ~5000 clones each and subjected to PCR amplification in a final 50 μl volume of 10 mM Tris-HCl, pH 8.3/50 mM KCl/2 mM $MgCl_2$/0.2 mM each dNTP/2 μM each A312 and A313 primers. Reactions were heated to 94°C for 5 min to ensure denaturation of the template DNA and then held at the 40°C annealing temperature. 1.25 units AmpliTaq DNA polymerase was added and the samples were subjected to 30 cycles of extension at 72°C for 1 min, denaturation at 94°C for 30 sec, and annealing at 40°C for 30 sec followed by a final extension at 72°C for 10 min. 12 μl of the reaction products were analyzed as described in Example 4 ("Southern Blotting"). Bacterial transformants from positive pools were screened by colony hybridization (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) with ^{32}P-labeled A315 oligonucleotide using conditions described above for

Southern blot analysis until a single isolated bacterial clone was obtained.

EXAMPLE 8

5' RACE

Amplification and isolation of the upstream 5' ends of receptor cDNA was accomplished using the technique of rapid amplification of cDNA ends (Frohman et al., Proc. Natl. Acad. Sci. USA 85:8998, 1988; Frohman, M.A. PCR Protocols: A Guide to Methods and Applications (Innis et al. eds.) pgs. 28-38, Academic Press, San Diego, 1990; Frohman, M.A. *Amplifications* 5:11, 1990; Schuster et al., *Focus* 14:46, 1992). (5' RACE system; Gibco BRL, Gaithersburg, MD) following the manufacturer's protocol. Briefly, 100 ng rat nucleus accumbens poly(A)$^+$ RNA was converted to first strand cDNA using A333 gene-specific primer. Following degradation of the RNA template with RNase H and purification of the cDNA, homopolymeric dC tails were added with terminal deoxynucleotidyl transferase. An aliquot equivalent to 6% of the input poly(A)$^+$ RNA template was PCR amplified in a final 50 μl volume containing 10 mM Tris-HCl, pH 8.3/50 mM KCl/2.0 mM $MgCl_2$/0.2 mM each dNTP/0.4 μM oligo-dC tail specific anchor primer (supplied with the kit)/0.4 μM gene-specific primer A361. Reactions were denatured for 5 min at 94°C and then held at 80°C. 1.25 units AmpliTaq DNA polymerase was added followed by 40 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 3 min. The final step was a 10 min extension at 72°C.

Amplification products were purified from 1% low melting point agarose gel slices, subcloned into plasmid

vector pAMP1 and transformed into *E. coli* DH5α (CloneAmp System; Gibco BRL, Gaithersburg, MD).

EXAMPLE 9

CONSTRUCTION OF FULL-LENGTH RECEPTOR cDNAs

The first full-length receptor cDNA was constructed as follows. Two receptor DNA fragments were prepared by restriction endonuclease digestion and purification from 1% low melting point agarose gels: i) ~1.2kb *Pst*I-*Stu*I fragment from 5' RACE subclone pAMP1-L2; and ii) ~2.2kb *Stu*I-*Xba*I fragment from cDNA library clone pcDNAI-275.

Corresponding L2 and 175 receptor cDNA fragments were introduced into *Pst*I-*Xba*I linearized and phosphatased eukaryotic expression plasmid pcDNAI in a three part ligation. 155 ng vector was mixed with both cDNA fragments in a 1:2:2 molar ratio of vector:L2:175 in a final 20 μl volume of 66 mM Tris-HCl, pH 7.5/5 mM $MgCl_2$/1 mM DTE/1 mM ATP containing 5 units T4 DNA ligase. After overnight incubation at 12°C an additional 5 units T4 DNA ligase were added and incubation continued overnight at room temperature. Ligation products were introduced into *E. coli* MC1061/P3 by electroporation. Full-length cDNA construct pcDNAI-L2175-D20 contains a ~3.4 kb insert in the correct oreintation for expression.

The second full-length receptor cDNA was constructed in a similar manner. A ~1.3 kB <u>Pst</u>I-<u>Stu</u>I fragment from 5'RACE clone pAMP1-U3 and a ~2.2 kb <u>Stu</u>I-<u>Spe</u>I fragment from cDNA library clone pcDNAI-237 were isolated from 1% low melting point agarose gels. They were introduced into *Pst*I-*Xba*I linearized and phosphatased eukaryotic expression plasmid pcDNAI in a three part

ligation. 155 ng vector was mixed with both cDNA fragments in a 1:2:2 molar ratio of vector:U3:237 in a final 20 μl volume of 66 mM Tris-HCl, pH 7.5/5 mM $MgCl_2$/1 mM DTE/1 mM ATP containing 5 units T4 DNA ligase. After overnight incubation at 12°C an additional 5 units T4 DNA ligase were added and incubation continued overnight at room temperature. Ligation products were introduced into *E. coli* MC1061/P3 by electroporation. Full-length cDNA construct pcDNAI-U3237-A2 contains a ~3.5 kb insert in the correct orientation for expression.

EXAMPLE 10

TRANSIENT TRANSFECTION

COS-7 cells were seeded at 1 x 10^7 cells per T-150 tissue culture flask and grown overnight. The monolayer (~75% confluent) was then transfected with 14 μg plasmid DNA and 120 μg Lipofectin reagent (Gibco BRL) in 7.5 ml Optimem media (Gibco BRL) containing 5.5 μM 2-mercaptoethanol. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 4 hrs after which the transfection media was replaced with DMEM (low glucose; 1000 mg/L)/10% fetal bovine serum/2% L-glutamine/1% Penicillin-Streptomycin.

Cells were harvested 60 hrs post-transfection by scraping into ice cold PBS and pelleted by centrifugation at ~160 x g for 4 min at 4°C. The cell pellet was resuspended in ice cold 20 mM HEPES and disrupted twice for 15 sec using a Brinkmann polytron homogenizer on setting number 3. The homogenate was then centrifuged at 48,400 x g for 20 min at 4°C and the pellet resuspended in ice cold 20 mM HEPES by brief homogenization. Aliquots were flash frozen in a dry ice/ethanol bath and then stored at -80°C.

DNA was sequenced by the dideoxy chain-termination method (Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977) with modified phage T7 DNA polymerase (Sequenase; United States Biochemicals, Cleveland, OH).

EXAMPLE 11

RADIOLIGAND BINDING ASSAYS

Radioligand studies were carried out in 20 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma, St. Louis, MO), pH 7.4, containing 1.0 mg/ml BSA (Protease free, Fraction V, Sigma), 1.0 mg/ml bacitracin (Sigma), 5 μg/ml bestatin-HCl (Sigma), 1 μg/ml phosphoramidon (Sigma). Membranes from COS-7 cells transfected with C1a receptor (C1a membranes) were thawed and diluted to a concentration of approximately 1.3 μg protein/ml while membranes from COS-7 cells transfected with C1b receptor (C1b membranes) were thawed and diluted to 13 μg protein/ml for all binding studies. Transfected COS cells from Example 9 were used to prepare the membranes. Protein determinations were made using the Bradford assay (Biorad, Richmond, CA) with BSA as a control.

Competition curves were generated by measuring binding of 8 pM ^{125}I-iodotyrosyl-salmon calcitonin for C1a membrane assays and 20 pM ^{125}I-iodotyrosyl-salmon calcitonin for C1b membrane assays in the presence of 10^{-12}M to 10^{-6}M unlabeled ligands. Rat amylin, rat α-CGRP, salmon calcitonin, rat calcitonin and human calcitonin were purchased from Bachem (Torrance, CA); rat β-CGRP was purchased from Peninsula Labs, Inc. (Belmont, CA).

All assays were run in quadruplicate in a total volume of 200 μl. Incubations were carried out for 60 minutes in 96 well microtiter plates (Corning, Corning, New York) at 23°C. Incubations were terminated by rapid filtration, under vacuum, through glass filter fiber pads (Wallac, Gaithersburg, MD) pretreated with 0.3% polyethyleneimine (Sigma) using a cell harvester (Tomtec, Orange, CT). Filters were washed using ice cold phosphate buffered saline (PBS), pH 7.4. Filter pads were dried and counted on a Wallac 1205 Betaplate scintillation counter.

To generate saturation isotherms (shown in Figures 2 and 4), binding of ^{125}I-iodotyrosyl-salmon calcitonin (~2000 Ci/mmol; Amersham Corp., Arlington Heights, IL) was measured at concentrations varying from 1-150 pM to obtain total binding, and in the presence of 100 nM unlabeled salmon calcitonin to obtain nonspecific binding.

For competition studies (shown in Figures 6 and 7), data were fit to a 4-parameter logistic equation, with derivation of apparent inhibition constants (K_i) from IC_{50} values using the Cheng-Prusoff relationship (INPLOT 4.03, GraphPAD, San Diego, CA). Values represent means ± standard deviations from duplicate experiments.

Specific and saturable binding of ^{25}I-iodotyrosyl-salmon calcitonin to C1a membranes was observed at concentrations of 6-190 pM (Figure 2). Scatchard analysis of C1a saturation data yielded a dissociation constant (Kd) = 8.19 ± 0.31 pM and a binding site density (Bmax) = 3905 ± 1305 fmol/mg protein (mean ± SD, n=2) (Figure 3). Specific and saturable binding of ^{25}I-iodotyrosyl-salmon calcitonin to C1b membranes was observed at concentrations of 4-140 pM (Figure 4). Scatchard analysis of C1b saturation data

yielded a dissociation constant (Kd) = 47.8 ± 2.8 pM and a binding site density (Bmax) = 924 ± 30 fmol/mg protein (mean ± SD, n=2) (Figure 5).

Competition experiments determined that the C1a and C1b clones have a unique selectivity profile for a series of structurally-related peptides, with potency for salmon calcitonin >> rat amylin > rat CGRP-α,β >> rat, human calcitonin for C1a clone (Figure 6), and salmon calcitonin >> rat amylin, rat CGRP-α,β >> rat, human calcitonin for C1b clone (Figure 7). The potencies of rat CGRP-α,β were 3-4 fold higher in the C1a clone, while rat amylin and salmon calcitonin had potencies 5-6 fold higher in the C1a clone.

Table 1 below indicates the concentration of ligand which produces half-maximal inhibition (IC50) of ^{125}I-salmon calcitonin binding to membranes from COS cells expressing the indicated receptor. Results are means of IC50s measured in 2-3 separate experiments.

TABLE 1

INHIBITION OF ^{125}I-SALMON CALCITONIN BINDING

Peptide	IC50 (nM)	
	Rat C1a	Rat C1b
Salmon calcitonin	0.032	0.158
Rat amylin	51	398
Rat β-CGRP	100	355
Rat α-CGRP	178	468
Rat calcitonin	>1000	>1000
Human calcitonin	>1000	>1000

EXAMPLE 12

TISSUE-SPECIFIC DISTRIBUTION

OF C1a and C1b RECEPTORS

A Northern blot containing 2 μg of poly(A)$^+$ RNA from eight rat tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis) was obtained from Clonetech. It was probed with a 1244 bp HindIIIEarI coding region fragment from clone L2175-D20 (positions 380-1623) labeled by random priming to a specific activity of 1.6 x 10^9 cpm/μg. Hybridization conditions were 5X SSPE/5X Denhardt's/2% SDS/0.1 mg ml^{-1} denatured herring sperm DNA for 21 hours at 65°C. The blot was washed in 2X SSC/0.1% SDS at 42°C and exposed to Kodak XAR film at -80°C with intesifying screens. A band of about 4.4 kb was identified in brain and kidney. Similar results were obtained using a C1b DNA probe.

EXAMPLE 13

ADENYLATE CYCLASE COUPLING

Transfected COS cells were cultured in Dulbecco's Minimum Essential medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 1 mg glucose/ml, and 2 mM L-glutamine. Twenty-four hours after transfection (described in Example 10), cells were subcultured at 1 x 10^5 cells/0.2 ml medium/well in 96-well tissue culture plates (Corning Glass Works, Corning NY). Cells were maintained at 37°C and 5% CO_2/95% humidified air.

Stimulation of cyclic AMP (cAMP) production was performed as follows. Twenty-four hours after subculture, medium was replaced with 100 μl Dulbecco's phosphate

buffered saline (DPBS; Sigma) containing 0.1 mg BSA/ml and 0.05 mg isobutylmethyl xanthine/ml, pH 7.3. Cells were incubated for 20 minutes at 37°C in equilibrium with air. DPBS was aspirated and replaced with 50 μl fresh DPBS. Fifty μl of DPBS containing test substance(s) at 2x final concentrations were added and plates were incubated an additional 25 minutes. The response was halted by acidification with 25 μl 10% trichloracetic acid, followed by neutralization with 25 μl 0.8 M Tris (hydroxymethyl) aminomethane.

Immunoreactive cAMP in supernatants was acetylated and measured using a scintillation proximity assay (Amersham, Arlington Heights, IL). Prior to assay, supernatants were diluted 1:25 in assay buffer and cAMP was acetylated following a protocol provided by the manufacturer.

Results shown in Figure 8 demonstrate that salmon calcitonin at 10^{-6}M strongly stimulates cAMP synthesis in COS cells expressing either C1a or C1b receptors, but not in vector-transfected control cells. Transfection did not alter β-adrenegic receptor coupling to adenylate cyclase in COS cells, as seen by the equal responsiveness to isoproterenol in all three cell populations. These results indicate that C1a and C1b transcripts encode functional receptors that are positively coupled to adenylate cyclase. Stimulation of adenylate cyclase activity or inhibition of agonist-stimulated activity in C1a- or C1b-expressing cells can be taken to indicate receptor agonist or antagonist activity, respectively.

Other embodiments are within the following claims.

WE CLAIM:

1. An assay method for use in identifying, screening for, evaluating, or characterizing C1a or C1b receptor binding compounds which comprises the steps of,

(a) bringing together a test sample and a C1a or C1b receptor preparation, said test sample containing one or more test compounds, and said receptor preparation containing a C1a or C1b receptor protein;

(b) incubating said test sample and said receptor preparation under conditions which permit the binding a C1a or C1b binding ligand to said C1a or C1b receptor protein; and,

(c) identifying those test samples containing one or more test compounds which detectably bind to said C1a or C1b receptor.

2. The assay method of claim 1 which further comprises,

(d) screening said test samples which detectably bind to said C1a or C1b receptor for in vitro or in vivo stimulation or inhibition of C1a or C1b receptor-mediated activity; and,

(e) identifying those test samples which act as agonists or antagonists of a peptide selected from the group consisting of calcitonin, amylin, and CGRP.

3. The assay method of claim 1 wherein said calcitonin receptor protein comprises C1a.

4. The assay method of claim 1 wherein said central calcitonin receptor protein comprises C1b.

5. The assay method of claim 2 wherein said C1a or C1b receptor-mediated activity is appetite modulation.

6. The assay method of claim 2 wherein said Cla or Clb receptor-mediated activity is pain modulation.

7. The assay method of claim 2 wherein said Cla or Clb receptor-mediated activity is modulation of glucose metabolism.

8. The assay method of claim 1 wherein said receptor preparation comprises cloned Cla receptor.

9. The assay method of claim 1 wherein said receptor preparation comprises cloned Clb receptor.

10. The assay method of claim 5 wherein said Cla or Clb receptor preparation comprises membranes obtained from transfected cells which express the Cla receptor.

11. The assay method of claim 5 wherein said Cla or Clb receptor preparation comprises membranes obtained from transfected cells which express the Clb receptor.

12. The assay method of claim 5 wherein said Cla or Clb receptor preparation comprises cells transfected with Cla receptor cDNA.

13. The assay method of claim 5 wherein said Cla or Clb receptor preparation comprises cells transfected with Clb receptor cDNA.

14. The assay method of claim 1 wherein said test samples which detectably bind to said receptor protein are assayed by measuring the displacement of a labelled first ligand from said Cla or Clb receptor preparation by said test sample, and comparing the measured displacement of said labelled first ligand from said Cla or Clb receptor preparation by said test sample with the measured displacement of said labelled first ligand from said receptor preparation by one or more known second ligands.

15. The assay method of claim 14 wherein said labelled first ligand comprises a calcitonin.

16. The assay method of claim 14 wherein said test sample comprises an amylin agonist.

17. The assay method of claim 14 wherein said test sample comprises an amylin antagonist.

18. The assay method of claim 14 wherein said first ligand is labelled with a label selected from the group consisting of radioactive isotopes, nonradioactive isotopes, fluorescent molecules, chemiluminescent molecules, and biotinylated molecules.

19. The assay method of claim 15 wherein said calcitonin comprises salmon calcitonin.

20. The assay method of claim 19 wherein said salmon calcitonin comprises ^{125}I-salmon calcitonin.

21. The assay method of any of claims 14, 15, 16, 17 or 18 wherein said known second ligand or ligands are selected from the group consisting of an amylin, a calcitonin, an α-CGRP, and a β-CGRP.

22. The assay method of any of claims 14, 15, 16, 17 or 18 wherein said known second ligand or ligands are selected from the group consisting of human amylin, dog amylin, rat amylin, human calcitonin, rat calcitonin, eel calcitonin, salmon calcitonin, human α-CGRP, human β-CGRP, rat α-CGRP, and rat β-CGRP.

23. The assay method of claim 14, wherein said test sample contains more than one test compound, which further comprises the steps of,

(d) preparing two or more additional test samples from said test sample, said additional test samples being characterized in that they contain a lesser number of test compounds than said test sample from which they were prepared; and,

(e) repeating steps (a)-(d) as many times as desired or as required until the test compound or compounds which bind to said Cla or Clb receptor have been identified.

24. The assay method of claim 2 wherein said test samples which detectably bind to said Cla or Clb receptor protein are assayed by measuring the displacement of a labelled first ligand from said Cla or Clb receptor preparation by said test sample, and comparing the measured displacement of said labelled first ligand from said Cla or Clb receptor preparation by said test sample with the

measured displacement of said labelled first ligand from said C1a or C1b receptor preparation by one or more known second ligands.

25. The assay method of claim 24, wherein said test sample contains more than one test compound, which further comprises the steps of,

(f) preparing two or more additional test samples from said test sample, said additional test samples being characterized in that they contain a lesser number of test compounds than said test sample from which they were prepared; and,

(g) repeating steps (a)-(f) as many times as desired or as required until the test compound or compounds which bind to said C1a or C1b receptor have been identified.

26. The assay method of any of claims 1, 2, 14 or 24 wherein said test sample comprises one or more known test compounds.

27. The assay method of any of claims 1, 2, 14 or 24 wherein said test sample comprises one or more unknown compounds.

28 An assay method for evaluating one or more receptor binding characteristics sought to be determined for a known or candidate calcitonin or amylin or CGRP agonist or antagonist compound, which comprises the steps of,

(a) bringing together a test sample and a C1a or C1b receptor preparation, said test sample containing one or

more test compounds, and said receptor preparation containing a C1a or C1b receptor protein;

(b) incubating said test sample and said receptor preparation under conditions which permit the binding a C1a or C1b binding ligand to said C1a or C1b receptor protein; and

(c) assessing or measuring the ability of said compound to compete against a labelled ligand for binding to said C1a or C1b receptor preparation.

29. The assay method of claim 28 which further comprises the steps of:

(d) assessing or measuring the ability of said test sample to compete against said labelled ligand for binding to an amylin receptor preparation, said amylin receptor preparation containing an amylin receptor protein which binds amylin; and/or,

(e) assessing or measuring the ability of said compound to compete against said labelled ligand for binding to a CGRP receptor, said CGRP receptor preparation containing a CGRP receptor protein which binds CGRP; and,

(f) determining the receptor binding characteristic sought to be determined for said test sample.

30. The assay method of claim 28 or 29 wherein said binding characteristic sought to be determined for said compound is C1a or C1b receptor binding affinity.

31. The assay method of claim 28 or 29 wherein said binding characteristic sought to be determined for said compound is Cla or Clb receptor binding specificity.

32. An assay method for determining the presence or amount of a Cla or Clb receptor binding compound in a test sample to be assayed for said compound, which comprises the steps of,

(a) bringing together said test sample to be assayed and a Cla or Clb receptor preparation, said Cla or Clb receptor preparation containing a Cla or Clb receptor protein;

(b) measuring the ability of said test sample to compete against a labelled ligand for binding to said Cla or Clb receptor preparation; and/or,

(c) relating the amount of Cla or Clb receptor binding compound in said test sample with the amount of Cla or Clb receptor binding compound measured for a control sample in accordance with steps (a) and (b), said control sample being known to be free of any Cla or Clb receptor binding compound; and/or

(d) relating the amount of Cla or Clb receptor binding compound in said test sample with the amounts of Cla or Clb receptor binding compound measured for control samples containing known amounts of Cla or Clb receptor binding compound in accordance with steps (a) and (b), to determine the presence or amount of Cla or Clb receptor binding compound in said test sample, or

33. A method of producing monoclonal antibodies that bind to a Cla or Clb receptor, which comprises the steps of,

(a) immunizing an animal with a Cla or Clb receptor preparation comprising a Cla or Clb receptor protein or a portion thereof;

(b) recovering B lymphocytes from said immunized animals;

(c) fusing said recovered B lymphocytes with malignant cells to produce hybridomas;

(d) recovering hybridomas that produce antibodies that bind said Cla or Clb receptor; and,

(e) recovering antibodies from one or more hybridomas selected in step (d).

34. A method of producing antibodies against a Cla or Clb receptor, which comprises the steps of,

(a) immunizing an animal with a Cla or Clb receptor preparation comprising a Cla or Clb receptor protein;

(b) selecting those animals whose sera contain anti-Cla or anti-Clb receptor antibodies; and,

(c) recovering sera containing anti-Cla or anti-Clb receptor antibodies from said selected animals.

35. A method for separating Cla or Clb receptor binding compounds from a sample, which comprises the steps of,

(a) bringing together said sample and a Cla or Clb receptor preparation, said Cla or Clb receptor preparation comprising Cla or Clb receptor protein molecules bound to a solid carrier; and

(b) separating any C1a or C1b receptor binding compound which is bound to said C1a or C1b receptor preparation from the remainder of said test sample which is unbound.

36. A method for screening a biological substance for the presence of C1a or C1b receptors, which comprises the steps of,

(a) bringing together said biological substance with first C1a or C1b receptor binding compound;

(b) bringing together said biological substance with a second C1a or C1b receptor binding compound;

(c) optionally bringing together said biological substance with one or more additional C1a or C1b receptor binding compounds; and,

(d) determining the relative binding affinities of said C1a or C1b receptor binding compounds for receptors in said biological substance.

37. The method of claim 36 wherein said biological substance comprises a cell line.

38. The method of claim 36 wherein said C1a or C1b receptor binding compounds are selected from the group consisting of the amylins, the calcitonins, the α-CGRPs, and the β-CGRPs.

39. Purified C1a receptor.

40. Purified C1b receptor.

41. Purified nucleic acid encoding a C1a receptor.

42. Purified nucleic acid encoding a C1b receptor.

43. Cells transfected with nucleic acid encoding a C1a receptor.

44. Cells transfected with nucleic acid encoding a C1b receptor.

45. The cells of either of claims 43 or 44 which are bacteria.

46. The cells of claim 45 wherein said bacteria are E. coli.

47. A vector containing nucleic acid encoding a C1a receptor.

48. A vector containing nucleic acid encoding a C1b receptor.

49. Cells transfected with a vector containing nucleic acid encoding a C1a receptor.

50. Cells transfected with a vector containing nucleic acid encoding a C1b receptor.

51. The cells of either of claims 49 or 50 which are COS-7 cells.

FIGURE 1

```
Clb (U3237-A2)     MRFLLLNRFTLLLLLLLVSPTPVLQAPTNLTDSGLDQEPFLYLVGRKKLLDA
Cla (L2175-D20)    MRFLLLNRFTLLLLLLLVSPTPVLQAPTNLTDSGLDQEPFLYLVGRKKLLDA

                                       E1
Clb (U3237-A2)     QYKCYDRIQQLPPYEGEGPYCNRTWDGWMCWDDTPAGVMSYQHCPDYFPDF
Cla (L2175-D20)    QYKCYDRIQQLPPYEGEGPYCNRTWDGWMCWDDTPAGVMSYQHCPDYFPDF

Clb (U3237-A2)     DPTEKVSKYCDENGEWFRHPDSNRTWSNYTLCNAFTPDKLHNAYVLYYLAL
Cla (L2175-D20)    DPTEKVSKYCDENGEWFRHPDSNRTWSNYTLCNAFTPDKLHNAYVLYYLAL

                       TM1             I1                   TM2
Clb (U3237-A2)     VGHSMSIAALIASMGIFLFFKNLSCQRVTLHKNMFLTYILNSIIIIIHLVE
Cla (L2175-D20)    VGHSMSIAALIASMGIFLFFKNLSCQRVTLHKNMFLTYILNSIIIIIHLVE

                                         E2
Clb (U3237-A2)     VVPNGDLVRRDPMHIFHHNTYMWTMQWELSPPLPLSAHEGKMDPHDSEVIS
Cla (L2175-D20)    VVPNGDLVRRDP---------------------------------------IS

                           TM3                  I2              TM4
Clb (U3237-A2)     CKILHFFHQYMMACNYFWMLCEGIYLHTLIVMAVFTEDQRLRWYYLLGWGF
Cla (L2175-D20)    CKILHFFHQYMMACNYFWMLCEGIYLHTLIVMAVFTEDQRLRWYYLLGWGF

                                 E3                         TM5
Clb (U3237-A2)     PIVPTIIHAITRAVYYNDNCWLSTETHLLYIIHGPVMAALVVNFFFLLNIV
Cla (L2175-D20)    PIVPTIIHAITRAVYYNDNCWLSTETHLLYIIHGPVMAALVVNFFFLLNIV

                          I3                             TM6            E4
Clb (U3237-A2)     RVLVTKMRQTHEAEAYMYLKAVKATMVLVPLLGIQFVVFPWRPSNKVLGKI
Cla (L2175-D20)    RVLVTKMRQTHEAEAYMYLKAVKATMVLVPLLGIQFVVFPWRPSNKVLGKI

                           TM7                          I4
Clb (U3237-A2)     YDYLMHSLIHFQGFFVATIYCFCNHEVQVTLKRQWAQFKIQWSHRWGRRRR
Cla (L2175-D20)    YDYLMHSLIHFQGFFVATIYCFCNHEVQVTLKRQWAQFKIQWSHRWGRRRR

Clb (U3237-A2)     PTNRVVSAPRAVAFAEPGGLPIYICHQEPRNPPVSNNEGEEGTEMIPMNVI
Cla (L2175-D20)    PTNRVVSAPRAVAFAEPGGLPIYICHQEPRNPPVSNNEGEEGTEMIPMNVI

Clb (U3237-A2)     QQDSSA
Cla (L2175-D20)    QQDSSA
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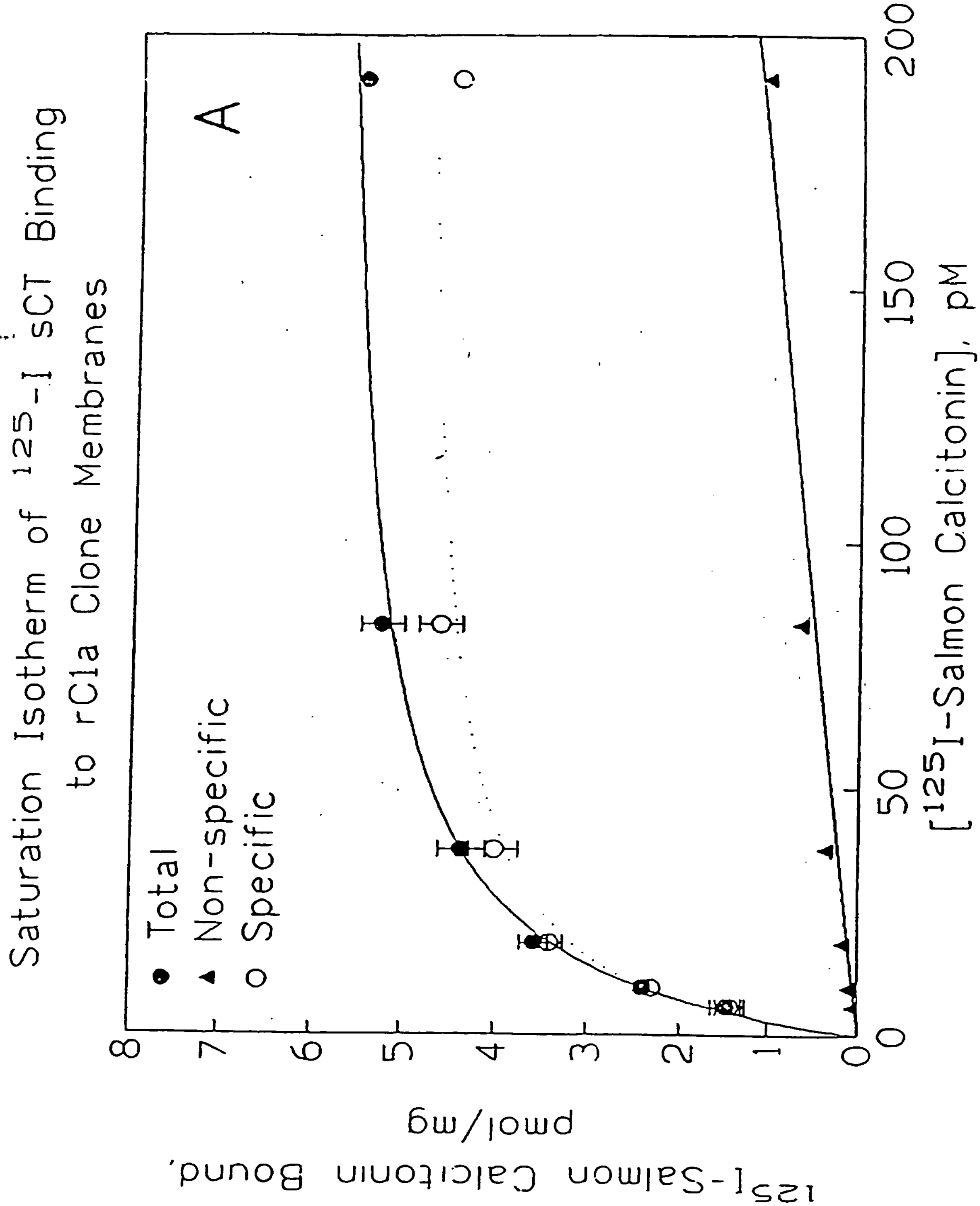

Saturation Isotherm of 125-I sCT Binding
to rCla Clone Membranes
A
Total
Non-specific
Specific
125I-Salmon Calcitonin Bound,
pmol/mg
0
1
2
3
4
5
6
7
8
0
50
100
150
200
[125I-Salmon Calcitonin], pM

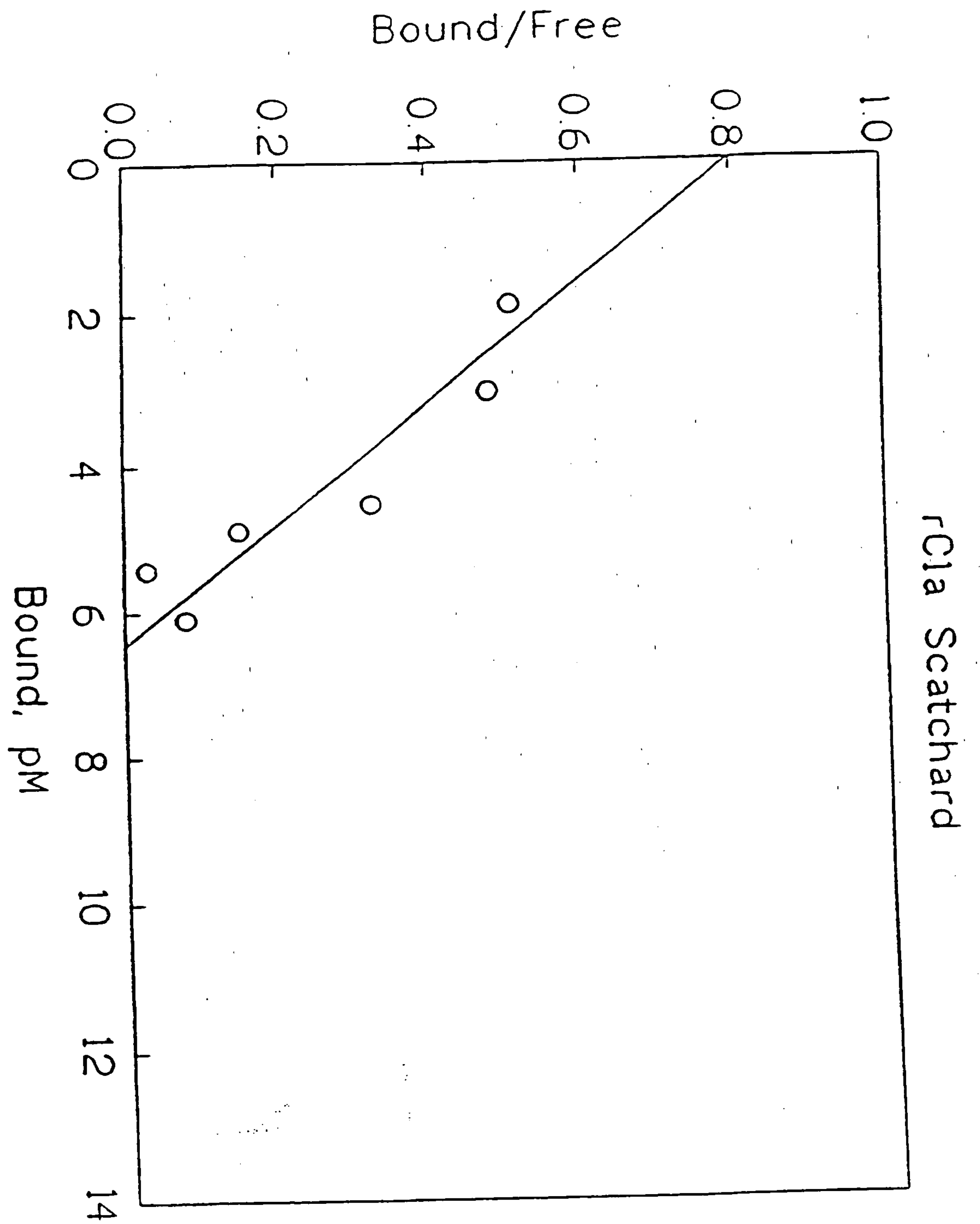
rCla Scatchard
Bound/Free
0.0
0.2
0.4
0.6
0.8
1.0
Bound, pM
0
2
4
6
8
10
12
14

Figure 3

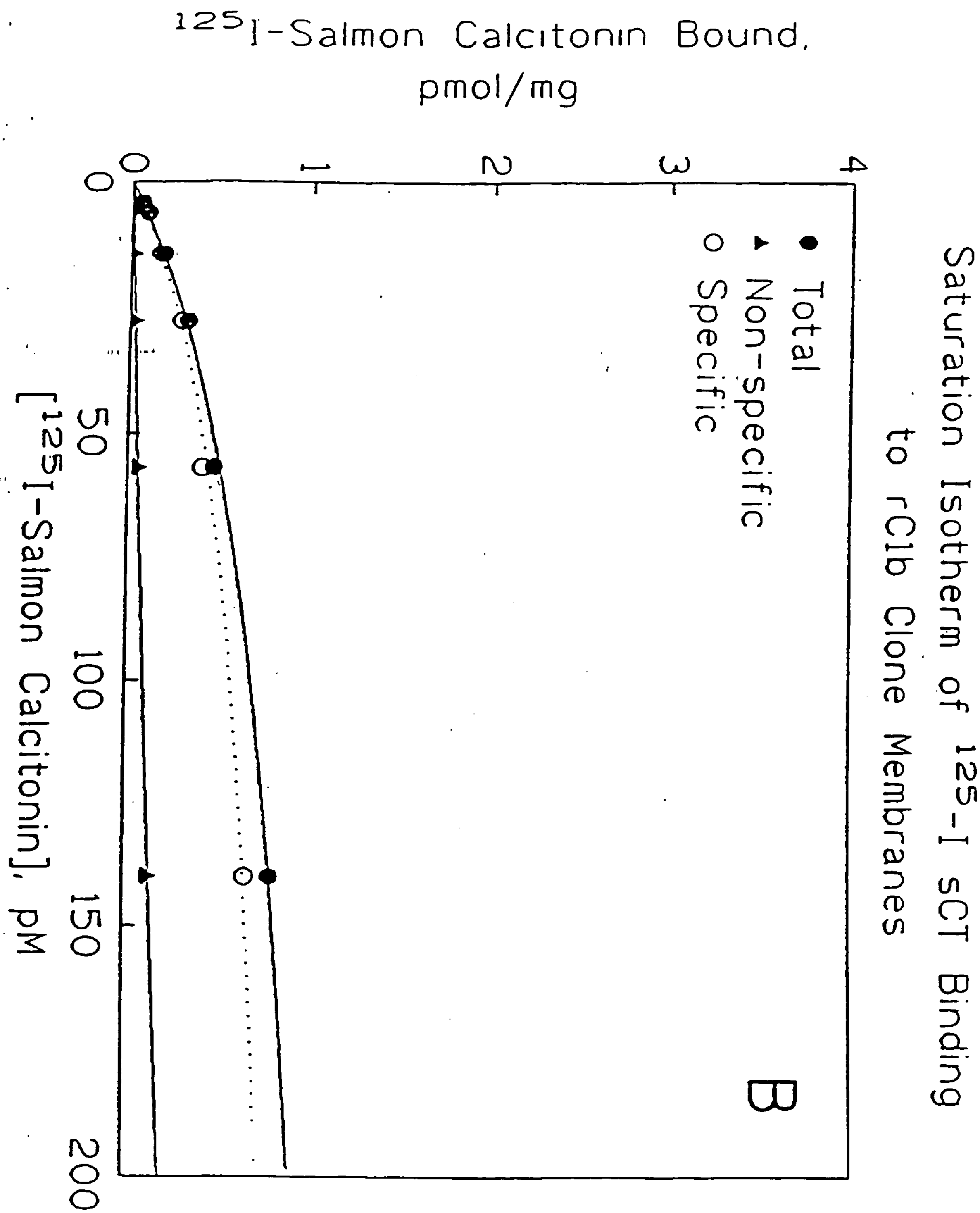
Saturation Isotherm of 125-I sCT Binding
to rC1b Clone Membranes
B
Total
Non-specific
Specific
125I-Salmon Calcitonin Bound, pmol/mg
0
1
2
3
4
0
50
100
150
200
[125I-Salmon Calcitonin], pM

Figure 4

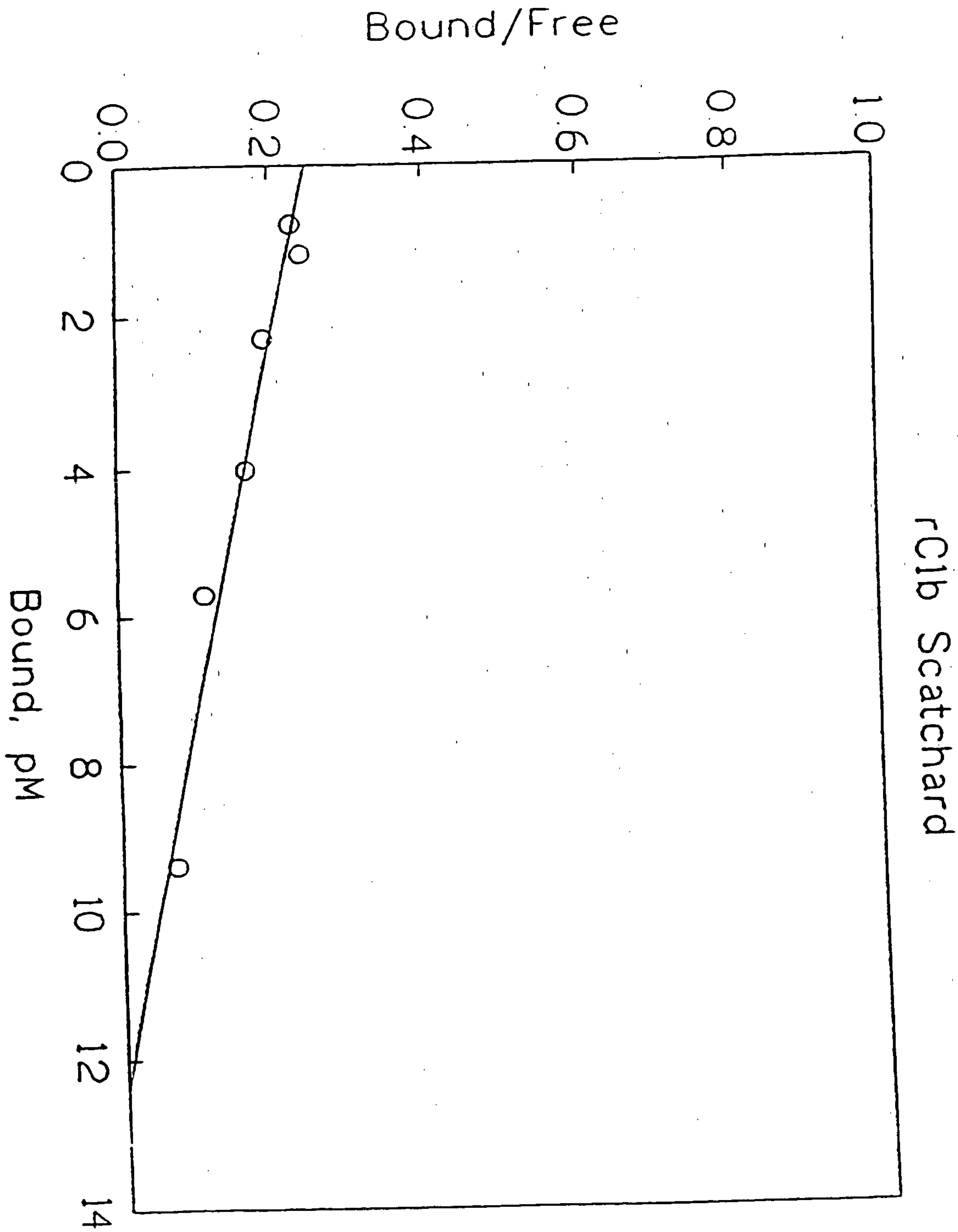
rC1b Scatchard
Bound/Free
0.0
0.2
0.4
0.6
0.8
1.0
Bound, pM
0
2
4
6
8
10
12
14

Figure 5

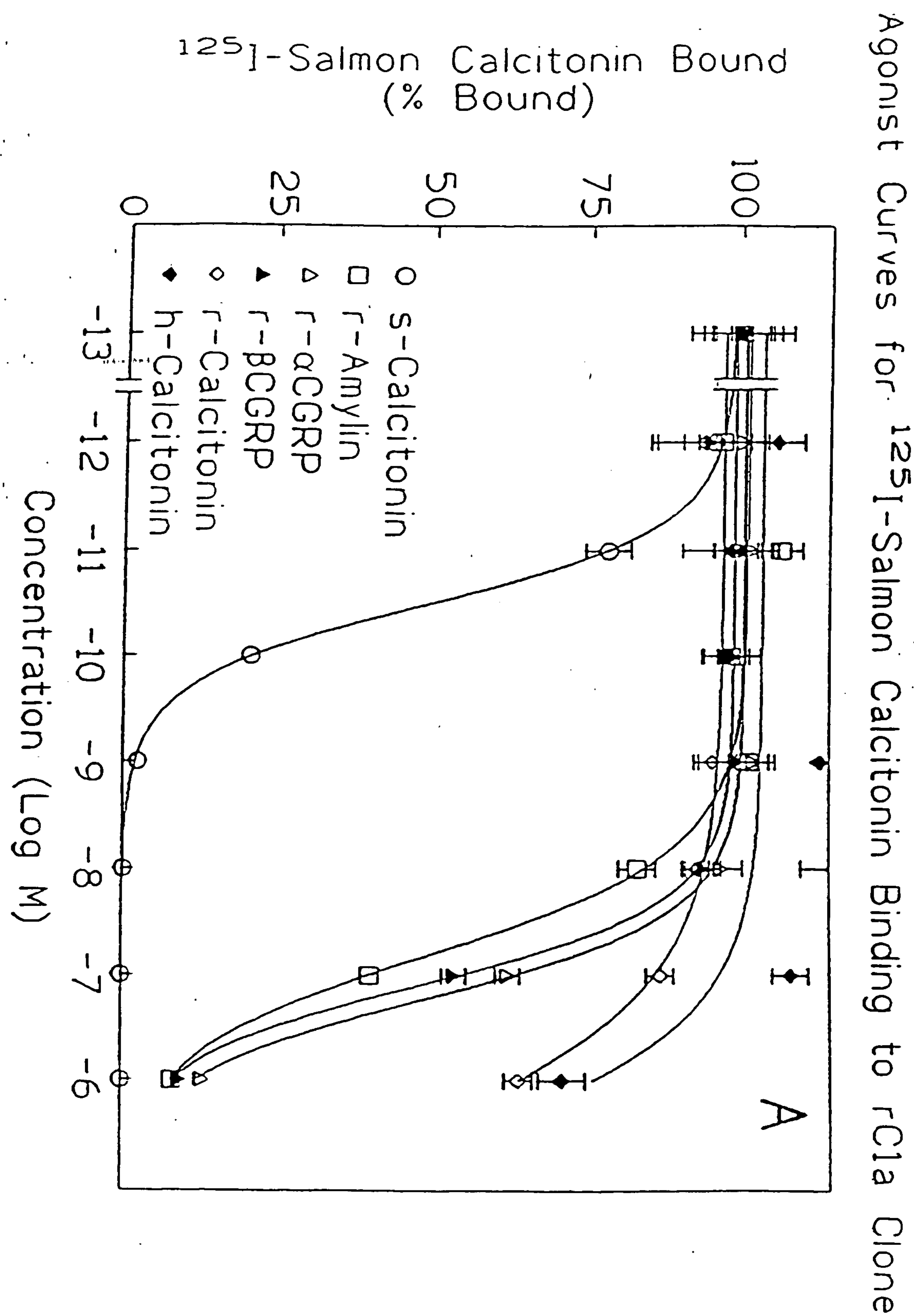
Agonist Curves for ^{125}I-Salmon Calcitonin Binding to rC1a Clone
^{125}I-Salmon Calcitonin Bound
(% Bound)
100
75
50
25
0
s-Calcitonin
r-Amylin
r-αCGRP
r-βCGRP
r-Calcitonin
h-Calcitonin
A
-13
-12
-11
-10
-9
-8
-7
-6
Concentration (Log M)

Figure 6

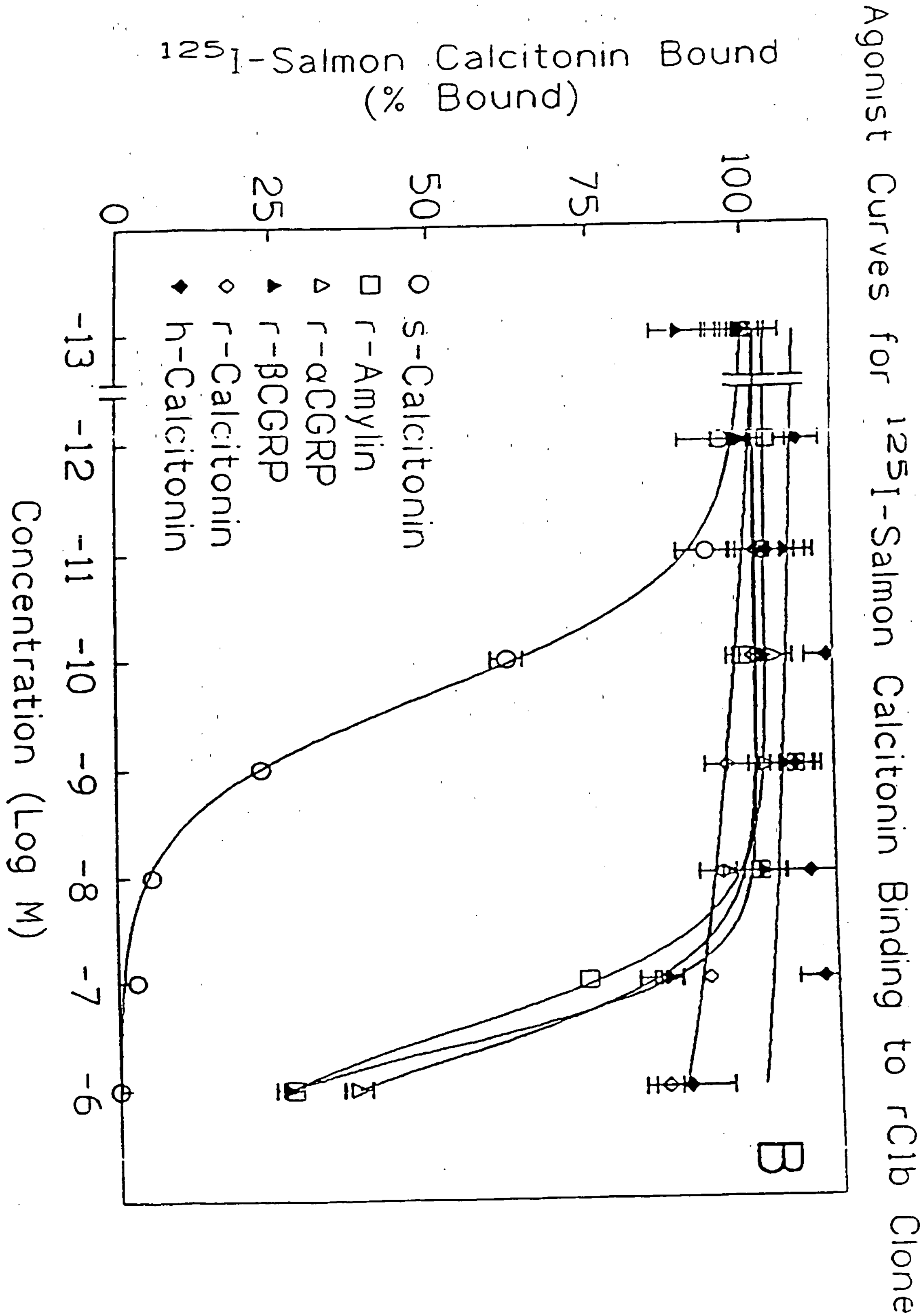
Agonist Curves for 125I-Salmon Calcitonin Binding to rC1b Clone
125I-Salmon Calcitonin Bound
(% Bound)
100
75
50
25
0
s-Calcitonin
r-Amylin
r-αCGRP
r-βCGRP
r-Calcitonin
h-Calcitonin
B
-13
-12
-11
-10
-9
-8
-7
-6
Concentration (Log M)

Figure 7

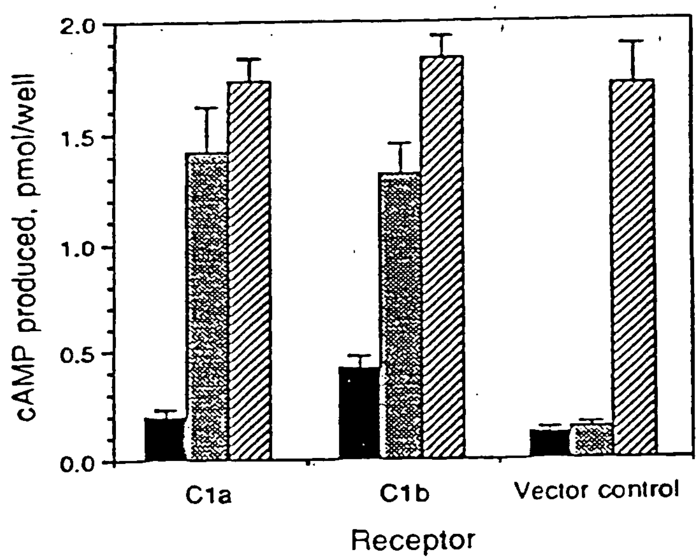
2.0
1.5
1.0
0.5
0.0
cAMP produced, pmol/well
C1a
C1b
Vector control
Receptor

FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03248

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/7.21, 240.2 252.33, 320.1; 436/518, 546, 548; 424/9; 530/350, 413

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG; PIR 40; SWISS-PROT 28; A-GENESEQ 14;

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P --- Y,P	FEBS LETTERS, Volume 325, Number 3, issued July 1993, K. Albrandt et al, "Molecular Cloning of Two Receptors from Rat Brain with High Affinity for Salmon Calcitonin", pages 225-232, see entire document.	1-28, 30-32, 36-51 ------- 29, 33-35
Y,P	US, A, 5,264,372 (BEAUMONT ET AL) 23 NOVEMBER 1993, see entire document, especially Col 12-13 and 15.	23, 25, 29, 33-34
Y	METHODS IN ENZYMOLOGY, Volume 70, issued 1980, Maurer et al, "Proteins and Peptides as Antigens", pages 49-70, see entire document, especially page 50.	33-35

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
01 JULY 1994

Date of mailing of the international search report
JUL 19 1994

Name and mailing address of the ISA/US
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Facsimile No. (703) 305-3230

Authorized officer
D. Vegza for
JAMES L. GRUN, PH.D.
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.
PCT/US94/03248

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	A.M. CAMPBELL, "MONOCLONAL ANTIBODY TECHNOLOGY. THE PRODUCTION AND CHARACTERIZATION OF RODENT AND HUMAN HYBRIDOMAS", published 1987 by ELSEVIER SCIENCE PUBLISHERS B.V. (AMSTERDAM), see page 29.	33-35
X ----- Y	SCIENCE, Volume 254, issued 15 November 1991, Lin et al, "Expression Cloning of an Adenylate Cyclase-Coupled Calcitonin Receptor", pages 1022-1024, see entire document, especially page 1022, Col 2-3, and Figures 1 and 3.	1, 3-4, 8-15, 18-19, 26, 28, 30, 39-51 ------- 23, 25, 29, 33-35
X ---- Y	SCIENCE, Volume 227, issued 15 March 1985, D. Goltzman et al, "Interaction of Calcitonin and Calcitonin Gene-Related Peptide at Receptor Sites in Target Tissues", pages 1343-1345, see entire document, especially Figures 1 and 2.	1-4, 14-22, 24, 26-28, 30-32, 36, 38 ------- 23, 25, 29
X ---- Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 78, issued August 1986, G.C. Nicholson et al, "Abundant Calcitonin Receptors in Isolated Rat Osteoclasts. Biochemical and Autoradiographic Characterization.", pages 355-360, see entire document, especially paragraph bridging pages 355-356.	1-4, 14-22, 24, 26-28, 30-32, 36-38 ------- 23, 25, 29
X ---- Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 90, issued November 1992, A. H. Gorn et al, "Cloning, Characterization, and Expression of a Human Calcitonin Receptor from an Ovarian Carcinoma Cell Line", pages 1726-1735, see entire document.	1-4, 8-22, 24, 26-28, 30-32, 36-51 ------- 23, 25, 29, 33-35

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07H 21/04; C07K 13/00, 17/02; C12N 1/21, 5/10, 15/70; G01N 33/543, 33/566, 33/577, 33/74

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.1; 435/7.21, 240.2 252.33, 320.1; 436/518, 546, 548; 424/9; 530/350, 413